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(54) Title: SCREENING FOR DISORDERS OF SEROTONERGIC DYSFUNCTION			
(57) Abstract <p>Three novel alleles of the serotonin transporter gene are disclosed and shown to be effective markers for screening and diagnosis of migraine and psychiatric disorders. The sequences of the alleles are given. Methods for <i>in vitro</i> screening of individuals using DNA taken from blood samples are taught.</p>			

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SCREENING FOR DISORDERS OF SEROTONERGIC DYSFUNCTION

The present invention relates to a method of screening for and for diagnosis of psychiatric disorders and other disorders of serotonergic function, for example migraine.

Serotonin (5-hydroxytryptamine or 5-HT) is known to be involved in brain function and activity. The serotonin transporter (also known as 5-HTT) has been targeted using highly selective drugs to effectively treat depressive illness and anxiety disorders (see Anderson et al, J Psychopharmacol 1994 8; 238-249).

The structure of the rat serotonin transporter cDNA was published in 1991 (Blakely et al, Nature 1991 354, 66-70; and Hoffman et al, Science 1991 254, 578-580) and US Patent No 5,418,162 is directed to the sequence of the cDNA for the rat serotonin transporter and its use as an oligonucleotide probe which could be used as a PCR extension primer. The corresponding human cDNA was reported by Lesch et al, Journal of Neural Transmission 91; 68-73 1993 and separately by Ramamoorthy et al, in Proceedings of the National Academy of Sciences, USA, 19; 2542-2546 1993.

1 The structure and arrangement of the human serotonin
2 transporter gene was first published in 1994 by Lesch
3 et al (Journal of Neural Transmission 95; 157-162).
4 The authors noted the existence of a "17bp repetitive
5 element" as a variable number tandem repeat (VNTR)
6 which occurred in the second intron of the gene. The
7 sequence data for the VNTR is available in the
8 Genbank/EMBL databases under accession number X76754
9 and is reproduced as part of Figure 1. Lesch et al
10 noted that the majority of the chromosomes examined had
11 either 10 or 11 copies of the repeat and for such
12 samples the frequency of the 10 VNTR sequence was 0.47
13 with 41% of individuals displaying heterogeneity. It
14 was speculated that the number of repeats could
15 possibly play a role in the pathogenesis of
16 neuropsychiatric illness. To date no evidence has been
17 reported which definitively links the VNTR sequences
18 with any particular function.

19
20 The human serotonin transporter gene is localised to
21 chromosome 17q11.1-q12. (see Ramamoorthy et al 1993
22 supra) and to date there is no published evidence for
23 genetic linkage of any affective disorder to this part
24 of the genome. Current data indicates that, while
25 there is a genetic basis for psychiatric disorders such
26 as anxiety and depression, and also for migraine, there
27 is no evidence which convincingly demonstrates an
28 underlying molecular basis for genetic susceptibility
29 in either case.

30
31 For example, a study made by Lesch et al in 1995
32 (Biological Psychiatry 37; 215-223) in which 17
33 patients suffering from major depressive or bipolar
34 disorder were screened for mutations in the serotonin
35 transporter cDNA sequence showed no difference compared
36 to the four controls.

1 The studies leading to the present invention have
2 surprisingly found 3 alleles of the VNTR region in
3 intron 2 of the serotonin transporter gene. The 3
4 alleles located are all novel and are designated
5 STin2.9, STin2.10 and STin 2.12 containing 9, 10 and 12
6 copies of the VNTR repeat, respectively. The third
7 allele (STin 2.10) containing 10 copies of the repeat
8 differs from that described previously by Lesch et al
9 (1994, supra). No individuals possessing 11 copies of
10 the repeat were identified.

11
12 The frequencies of the different allele forms were
13 compared between the control group and groups having a
14 major affective disorder. There was a significant
15 difference between the control and affective disorder
16 groups. In particular the presence of the STin2.9
17 allele was found to be significantly associated with
18 affective disorder and was most common in unipolar
19 patients. This is the first time that a genetic
20 variation at the level of DNA sequence in a candidate
21 gene has been positively associated with affective
22 disorders.

23
24 Thus, the present invention provides the novel alleles
25 STin2.9, STin2.10 and STin2.12. The sequence of each
26 of the alleles STin2.9, STin2.10 and STin2.12 are
27 presented in Figure 1, labelled accordingly and
28 compared to the 10 repeat sequence reported by Lesch et
29 al, 1994, supra. The present invention also provides a
30 polynucleotide having a sequence substantially as set
31 out in Figure 1 for the alleles STin2.9, STin2.10 or
32 STin2.12 or a part thereof. The present invention
33 encompasses these alleles or the polynucleotides in
34 vectors and in transformed cells. Likewise the present
35 invention incorporates the use of such alleles,
36 polynucleotides, derivatives or parts thereof in

1 genetic engineering procedures (for example as probes
2 for PCR).

3

4 In a further aspect, the present invention provides a
5 cell line (preferably a mammalian cell line and
6 particularly a human cell line) comprising at least one
7 of the alleles STin2.9, STin2.10 or STin2.12 or a
8 polynucleotide having a sequence substantially as set
9 out for one of those alleles in Figure 1.

10

11 The sequences of alleles STin2.9, STin2.10 and STin2.12
12 are also presented in the sequence listing as SEQ ID
13 Nos 1, 2 and 3 respectively.

14

15 Generally the allele or polynucleotide will be located
16 in intron 2 of at least part of the serotonin
17 transporter gene.

18

19 Likewise the present invention includes a transgenic
20 animal which contains novel alleles and sequences
21 according to the present invention. Generally the
22 transgenic animal will be a mammal, especially a
23 laboratory animal for example a rat or mouse.

24

25 The cell line (which may be a transformed cell line)
26 and transgenic animal according to the present
27 invention may each independently be used as a model to
28 evaluate potential agents which may be effective for
29 combatting psychiatric disorders and other disorders of
30 serotonergic function, for example migraine.

31

32 There exists in the art numerous publications
33 describing how to form such vectors, transformed cell
34 and transgenic animals. Reference may be made to
35 "Principles of Gene Manipulation" Old and Primrose, 5th
36 edition, 1995, Blackwell Scientific Publications (and

1 the references therein) as providing a general
2 background to the subject.

3
4 In a yet further aspect, therefore, the present
5 invention provides a method of evaluating agents for
6 the ability to influence the expression of the
7 serotonin transporter, said method comprising exposing
8 a cell line or transgenic animal as described above to
9 said agent and determining the effect of said agent on
10 the expression of the serotonin transporter.

11
12 In another aspect the present invention provides a
13 method of diagnosis of psychiatric disorders, said
14 method comprising analysing the number of VNTR repeats
15 in the second intron of the serotonin transporter gene.

16
17 In a further aspect, the present invention provides a
18 method of diagnosis of an individual's susceptibility
19 to migraine, said method comprising analysing the
20 number of VNTR repeats in the second intron of the
21 serotonin transporter gene.

22
23 Advantageously such methods of the present invention
24 will look particularly for the alleles STin2.9,
25 STin2.10 and STin2.12, and especially for STin2.9.

26
27 Viewed from a further aspect the present invention
28 provides a method of screening individuals for the
29 potential to develop a psychiatric disorder or to
30 suffer from migraine, said method comprising analysing
31 the number of VNTR repeats in the second intron of the
32 serotonin transporter gene.

33
34 Advantageously such methods of the present invention
35 will look particularly for the alleles STin2.9,
36 STin2.10 and STin2.12, and especially for STin2.9.

1 Particular psychiatric disorders which may be diagnosed
2 and screened for using the methodology as mentioned
3 above include, from the DSM-IV taxonomy, mood
4 disorders, anxiety disorders and personality disorders.
5 The particular disorders of interest (DSM-IV codes in
6 parentheses) are depressive disorders (296.XX, 296.2X,
7 296.3X, 300.4, 311), and particular anxiety disorders
8 (300.01, 300.21, 300.22, 300.23, 300.3, 300.02,
9 300.00), personality disorders (301.83, 301.4) and
10 general medical disorders characterised by abnormal
11 serotonergic function including migraine and irritable
12 bowel syndrome. Thus, the invention may be used to
13 diagnose and screen for affective disorders, in
14 particular unipolar depressive illness, and related
15 anxiety disorders (for example panic disorder,
16 obsessional compulsive disorder), migraine and
17 irritable bowel syndrome.

18

19 The invention may also be useful in diagnosis of, or in
20 identifying propensity to dementia such as alzheimer's
21 disease, and to aggression, particularly that
22 associated with dementia, since it can be shown that
23 defective serotonin transmission in brain is linked to
24 these abnormalities.

25

26 Migraine is one of the most common neurological
27 disorders, affecting 16-23% of the general population
28 (Rasmussen BK et al Cephalagia 1992;12:221-28, and
29 Russell MB et al Int. J. Epidemiology 1995;24:612-18).
30 There are two main types of migraine. The first,
31 migraine without aura (MO; previously called common
32 migraine) is characterised by headache attacks lasting
33 4-72h. The headache is usually severe, unilateral,
34 pulsating, aggravated by physical activity, and
35 accompanied by nausea, vomiting, photophobia, and
36 phonophobia. In the second type, migraine with aura

1 (MA; previously classical migraine), the attack is
2 preceded by an aura i.e., reversible visual, sensory,
3 motor and/or aphasic symptoms. The ensuing headache
4 is very similar to that of MO (Rasmussen BK et al.
5 Cephalagia 1996;16:239-245).

6
7 The results of most family studies of migraine that use
8 segregation analysis have suggested that genetic
9 factors account for a significant degree of the
10 variance of MO and MA. Russell and colleagues (see
11 Neurology, 1993, 43 : 1369-73) have studied 121
12 individuals with MO and 72 individuals with MA in a
13 Danish population, diagnosed according to IHS criteria
14 and ascertained from the community using the Danish
15 Central Person Registry. They reported that, compared
16 with the general population, the first-degree relatives
17 of individuals with MO had a three-fold increase of MO,
18 while the first-degree relatives of individuals with MA
19 had a two-fold increase both of MO and of MA. Compared
20 with the general population, few spouses had either MO
21 or MA. This strongly suggested that MO and MA are
22 genetically determined although the study suffered from
23 the lack of direct interview of relatives.

24
25 A later, though similar study conducted by Russell &
26 Olesen (see BMJ, 1995, 311 : 541-4) the first-degree
27 relatives of individuals with migraine were
28 interviewed. They found that the first-degree
29 relatives of individuals with MO and 1.9 times the risk
30 of MO and 1.4 times the risk of MA. First-degree
31 relatives of individuals with MA had 3.8 times the risk
32 of MA and no increased risk of MO. The first-degree
33 relatives of screened controls had no increased risk of
34 MO or MA. Although a different pattern of results
35 emerged from those reported in the 1993 study (see
36 Russell et al 1993 supra), the results nevertheless

1 strongly suggest that MO and MA have a different
2 aetiology, and as they are based upon direct
3 neurological interview and examination of all the
4 relatives, are probably more reliable than the original
5 study. The genes contributing to genetic
6 susceptibility for MO and MA remain to be identified.

7
8 Mochi and colleagues (see Cephalagia, 1993, 13 : 389-
9 94) have performed segregation analysis on groups of
10 families with MO and MA. The resulting heritability
11 coefficients, a measure of the degree of concordance
12 among first-degree relatives, indicate a major genetic
13 component in both MO and MA, and were interpreted as
14 suggesting for MA, a possible multifactorial threshold
15 character, and for MO, the likely presence of a major
16 susceptibility gene with reduced penetrance.

17
18 A greater understanding of molecular migraine
19 mechanisms has come from the study of serotonin (5-HT)
20 and its receptor subtypes. One of the most important
21 initial strands of evidence implicating serotonin in
22 the pathogenesis of migraine was the claim that its
23 intravenous injection tends to reverse migrainous
24 headache. Further work in this field has shown that
25 during a migraine attack, platelet serotonin levels
26 decrease, urinary serotonin increases in some patients,
27 and 5-HIAA, a major metabolite of serotonin, may
28 increase. Other evidence suggesting a role for
29 serotonin is based on the observation that headache can
30 be precipitated by reserpine (which depletes neural
31 serotonin stores). In addition, it may be relieved by
32 selective 5-HT_{1D} agonists such as sumatriptan, and
33 blocked by treatment with methysergide (a serotonin
34 receptor antagonist).

35
36 There is striking similarity between the epidemiology

1 of migraine and that of depression, both disorders in
2 which serotonergic mechanisms have been implicated.
3 Major depression, like migraine, is a common disorder
4 with estimated lifetime prevalence ranging from 2-12%
5 for men and 5-25% for women, and it may be precipitated
6 by reserpine in susceptible subjects. In addition, low
7 levels of platelet serotonin and other abnormalities of
8 its metabolites have been shown. Both migraine and
9 depression show an efficacious response to treatment by
10 tricyclic and monoamine oxidase inhibiting
11 antidepressants, both having serotonergic activity.

12
13 Several studies have attempted to examine the
14 association between migraine and depression. A
15 clinical study by Merikangas and colleagues (see
16 Psychiatry Res, 1988, 2 : 119-29) yielded significant
17 associations between the two conditions. Systematic
18 studies of migraine and depression in community samples
19 have shown remarkable similarity in their reported
20 results (see Merikangas et al, 1988 supra; Merikangas
21 et al, Arch Gen Psychiatry, 1990, 47 : 849-53; and
22 Breslau et al, Psychiatry Res, 1991, 37 : 11-23). The
23 odds ratio (OR), which measures the degree of
24 association between the two disorders, was nearly
25 identical in these three studies (OR=3.5, 3.1, 3.6
26 respectively), confirming the clinical observation
27 regarding an association between migraine and
28 depression. Such co-morbidity may represent shared
29 risk or common aetiology, a possibility also suggested
30 by segregation analyses (see Merikangas et al, 1990,
31 supra). It is plausible, therefore, that serotonin
32 provides a common neurochemical basis for this
33 interaction.

34
35 In more detail the number of VNTR repeats occurring in
36 intron 2 of the serotonin transporter gene may be

1 determined in vitro from a sample taken from the
2 patient using technologies such as (for example)
3 polymerise chain reaction (PCR), heteroduplex analysis
4 and Southern blotting. Other methods include
5 comparative genome hybridisation (Methods in Enzymology
6 Rayburn, 1993, Vol 224, pages 204-212), single strand
7 conformational polymorphism analysis (see Lenk et al,
8 Neuromuscular Disorders 1994 4 : 411-418) and Ligase
9 Chain Reaction (see Jou et al, J Human Mutation 1995 5
10 : 86-93). Where a probe is required in these
11 techniques any sequence able to hybridise to the
12 sequences of interest may of course be used.

13
14 In a preferred aspect the present invention provides
15 methods of diagnosis and/or screening for psychiatric
16 disorders or for susceptibility to migraine, which
17 method comprises obtaining a sample from the individual
18 and screening the sample in vitro to look for the
19 number of VNTR repeats appearing in intron 2 of the
20 serotonin transporter gene. Where 9 repeats of the
21 VNTR are located it may be concluded that the
22 individual can be considered to be at risk of or
23 suffering from psychiatric disorders and the individual
24 may be treated accordingly. Where 12 repeats of the
25 VNTR are located it may be concluded that the
26 individual can be considered to be at risk of or
27 suffering from MO, whilst 9 repeats of the VNTR
28 suggests an increased risk of MA. The present
29 invention may be particularly of importance in aiding
30 accurate prescription needs, especially having regard
31 to the need for continuing therapy.

32
33 It may be convenient to conduct the methods of the
34 present invention on DNA extracted from a blood sample,
35 especially white blood cells. Any other physiological
36 sample may also be suitable; mention may be made of

1 body fluids containing DNA (such as saliva or blood)
2 and other non-fluid samples such as hair.

3

4 The present invention will now be illustrated with
5 reference to the following, non-limiting, examples.

6

7 Example 1

8

9 Subjects and Methods. The design of the study was
10 approved by the relevant Ethics Committee. Patients
11 with major affective disorder were recruited from the
12 inpatient and outpatient services of the Royal
13 Edinburgh Hospital. We planned to enter at least 80
14 patients and 160 controls into the study. 39 patients
15 with single or recurrent major depressive episodes and
16 44 patients with bipolar disorder were eventually
17 included. All fulfilled both the DSM IV criteria (see
18 American Psychiatric Association "Diagnosis and
19 Statistical Manual of Mental Disorders" 3rd edition,
20 revised, Washington DC, 1987) for major depressive
21 disorder or bipolar disorder and also the "probable"
22 Research Diagnostic Criteria (see Spitzer et al, Arch
23 Gen Psychiatry 1978 35: 773-782) according to the
24 Schedule for Affective Disorders and Schizophrenia
25 (Lifetime version)(SADS-L) (Endicott et al, Arch Gen
26 Psychiatry 1978, 35: 837-844) on interview and case
27 note evaluation by an experienced psychiatrist.

28

29 Controls came from two sources. A group of 122
30 anonymous control samples were obtained through the co-
31 operation of the local Blood Transfusion Service.
32 They were not screened for the presence of a personal
33 or family history of psychiatric disorder but met the
34 normal criteria for blood donation and so were taking
35 no regular psychotropic medication. A further group
36 of 71 volunteer controls were obtained from several

1 sources and was screened using a short questionnaire
2 based on sections of SADS-L to exclude affective
3 disorder, anxiety disorders other psychotic disorders
4 and alcohol problems both in the subjects themselves
5 and in first or second degree relatives. In addition,
6 all those who suffered from probable migraine or
7 irritable bowel syndrome, considered by some to be
8 "affective spectrum disorders" in which a serotonergic
9 mechanism has been implicated (see Hudson et al, Am J
10 Psychiatry 1990 147: 552-564) were excluded.

11

12 The mean ages of the patient and control groups were:
13 unipolar 43.4, bipolar 43.7, screened controls 47.2.
14 The sex ratios (female : male) were: unipolar (48.7 :
15 51.3), bipolar (47.0 : 53.0) and screened controls
16 (35.2 : 64.8).

17

18 DNA Isolation. Venous blood samples were frozen
19 immediately in dry ice and stored at -70°C. Genomic
20 DNA was isolated as described previously (see Smith et
21 al, Lancet 1992 339: 1375-1377). Briefly, 100µl of
22 whole blood was washed three times in TE buffer (10mM
23 Tris-HCl, pH8, 1mM EDTA), peripheral blood leucocytes
24 were harvested by centrifugation and re-suspended in
25 100µl lysis buffer (50mM KCl, 20mM Tris-HCl (pH 8.3),
26 2.5mM MgCl₂, 0.45% Nonidet P-40, 0.45% Tween 20)
27 containing 200µg ml⁻¹ Proteinase K. Lysis was completed
28 by incubation for 20 minutes at 55°C and the crude
29 lysates were diluted with an equal volume of sterile
30 distilled water and heated to 96°C for 10 minutes to
31 inactivate the proteinase. Samples were either used
32 immediately or stored at -20°C until required.

33

34 PCR of Intron 2. Target DNA (2-5µl of lysate) was
35 amplified by polymerase chain reaction using specific
36 oligonucleotide primers; 8224 (5'GTCAGTATCACAGGCTGCGAG)

1 and 8223 (5'-TGTTCTAGTCTTACGCCAGTG) whose sequences
2 appear in the sequence listing at SEQ ID Nos: 5 and 4
3 respectively. This primer pair amplifies the VNTR
4 region of intron 2 containing the 17bp repetitive
5 element as is illustrated in Figure 2. PCR was
6 carried out using 1.5U Taq polymerase (Promega), 100ng
7 of each primer, 200µM each of dATP, dCTP, dGTP, and
8 dTTP, 0.5% or 1.0% (v/v) DMSO and 1.5mM MgCl₂ in 1 x PCR
9 buffer (Promega) (50mM KCl, 10mM Tris-HCl (pH9), 0.1%
10 Triton X-100) in a final reaction volume of 50µl.
11 Thermal cycling was carried out in a Hybaid Omnigene
12 with a PCR profile starting with an initial strand
13 separation at 94°C for 4 minutes followed by 35-43
14 cycles of primer annealing at 60°C (20s),
15 polymerisation at 72°C (20s) and denaturation at 94°C
16 (30s). A final polymerisation step of 120s was
17 carried out to complete elongation of all amplified
18 strands. Amplified fragments were resolved on 5% non-
19 denaturing polyacrylamide gels and bands visualised by
20 ethidium bromide staining and UV transillumination
21 (Figure 3). The identity of the products was
22 confirmed by digestion with restriction enzymes HaeIII,
23 BstN I and Sma I and by direct sequencing.
24
25 Amplified fragments were separated on 2% agarose gels,
26 excised and purified by the Wizard PCR DNA purification
27 system (Promega). Sequencing was performed using the
28 Prism DyeDeoxy Terminator Cycle sequencing kit with one
29 of the primers used to generate the PCR product. The
30 sequencing reactions were performed in a Perkin Elmer
31 Cetus thermal cycler (30 cycles consisting of 30s at
32 96°C, 15s at 50°C and 4 min at 60°C). Unincorporated
33 nucleotides were removed by phenol/chloroform
34 extraction. Electrophoresis was carried out on an
35 Applied Biosystems model 373 STRETCH DNA Sequencer at a
36 constant power of 30W for 12 hours using a 4.75%

1 denaturing polyacrylamide gel.

2

3 Statistical Analysis. Patients were examined both as
4 separate unipolar and bipolar disorder groups and as a
5 combined group. Analysis was carried out on the raw
6 frequencies by the Chi squared test and by the Fisher
7 exact test (two tailed). These calculations were
8 performed using the Statistical Package for the Social
9 Sciences (Apple Mackintosh version 4.0). In addition
10 odds ratios and confidence limits were calculated by
11 standard methods.

12

13 Heteroduplex Analysis. PCR products were denatured for
14 3 minutes at 95°C and allowed to cool to 37°C over 30
15 minutes. Samples (5µl) were electrophoresed through
16 MDE Hydrolink gels (AT Biochem) at 800V overnight and
17 bands were visualised by silver staining.

18

19 Results.

20 Three alleles of the VNTR region in intron 2 of the
21 serotonin transporter gene were detected by PCR
22 followed by polyacrylamide gel electrophoresis. The
23 sequence data for the three alleles is presented in
24 Figure 1. By sequencing representative PCR products,
25 we identified three novel alleles (STin2.9, STin2.10
26 and STin2.12) containing, respectively, 9, 10 and 12
27 copies of the VNTR repeat. The third allele present
28 in our subjects (STin2.10) contained 10 copies of the
29 repeat and differed from that as described by Lesch et
30 al 1994, supra). We were unable to identify any
31 individuals possessing 11 copies of the repeat.

32

33 All chromosomes examined contained either 9, 10 or 12
34 copies of the 17bp repeat, with frequencies of 0.02,
35 0.40 and 0.58 respectively. The consensus sequence is:

36

GGCTGYGACCY(R)GRRTG

1 There was loss of the 12th base in 3 repeats. STin2.12
2 showed an additional 2 repeats in the area of
3 alternating 16 and 17bp motifs. 11 copies of the VNTR
4 were not seen in any of the PCR products analyzed here.
5 The third novel allele on the VNTR containing 9 copies
6 of the repeat is identical to STin2.10 except for the
7 loss of the 6th repeat.

8
9 There are some minor differences between some of the
10 repeats within the consensus sequence and the pattern
11 of repeats for the various alleles may be represented
12 as follows (see Figure 1):

13
14 STin2.12 A B C D E F D G D G D F
15 STin2.10 A B C D E F D G D F
16 STin2.9 A B C D E D G D F

17
18 Lesch A" B C D" E G D" G D" F
19 indicates that the repeat does not correspond exactly
20 to that of the novel repeats in the present invention.

21
22 It is interesting to note that in STin2.9 the 6th
23 repeat is a 16mer rather than a 17mer as in the other
24 two alleles of the present invention.

25
26 Since there was no significant difference in the
27 frequency of the three alleles between the screened and
28 BTS control groups, all further statistical comparisons
29 were made between the patient groups and the combined
30 control group.

31
32 There was a significantly higher frequency of genotypes
33 containing the STin2.9 allele in the unipolar group
34 compared to the control group ($P < 0.002$: Table 1, and
35 Figure 4). There was also a statistically significant
36 difference between the combined affective disorder

1 group and the control group in the frequency of
2 individuals carrying the STin2.9 allele ($P < 0.02$:
3 Table 1). These differences were significant in a
4 two-tailed Fisher's exact test at $P < 0.01$ and $P <$
5 0.05 , respectively. When allele frequencies were
6 considered, there remained a significant difference
7 between the unipolar and control groups ($\chi^2 = 9.87$,
8 $P < 0.01$: Table 1). In addition there appeared to be a
9 tendency for affected individuals to have allelic forms
10 with fewer VNTRs than control subjects ($\chi^2 = 9.56$, $P <$
11 0.05).

12
13 Odds ratios were calculated for the risk of affective
14 disorder if a single copy of the STin2.9 allele was
15 present. For the risk of unipolar disorder given a
16 single STin2.9 allele, the odds ratio was 6.95, with
17 95% confidence limits of 1.8-27.2 (Table 1).

18
19 Discussion

20 A dysfunction of the serotonergic system has long been
21 suspected in depression and other affective and anxiety
22 disorders but could not previously be definitely linked
23 to any defect thereof. Drug-free depressed patients
24 have been reported to have reduced serotonin
25 metabolites in CSF and postmortem brain tissue,
26 decreased plasma tryptophan concentrations and an
27 increase in the density of brain 5-HT₂ binding sites
28 (see Ins et al, Clin Chem 1994, 40: 288-295).

29
30 It is known that antidepressant drugs which act
31 specifically to block serotonin re-uptake have
32 comparable efficacy to tricyclic antidepressants and
33 monoamine-oxidase inhibitors which act on other
34 monoamine neurotransmitters as well as serotonin.
35 Many investigators have reported low numbers of
36 platelet and brain serotonin (5-HT) transporter sites

1 in drug-free depressed patients (see Boyer et al,
2 "Selective serotonin re-uptake inhibitors, Chichester :
3 John Wiley & Sons Ltd, 1991, pages 71-80 and references
4 cited therein). Our results suggest a mechanism by
5 which genetic variability in the serotonin transporter
6 gene may play a role in determining in susceptibility
7 to depression.

8
9 There are now several documented examples of
10 neuropsychiatric disorders caused by variations of
11 expansion of triplet repeats (see Ross et al, Trends
12 Neurosci 1993, 7:254-260) but few instances in which
13 VNTRs with longer repeating sequences confer
14 susceptibility to disease. The *IDDM2* locus, conferring
15 susceptibility to type 1 diabetes, has been mapped to a
16 14-15 bp VNTR located between the tyrosine hydroxylase
17 and insulin genes on chromosome 11p15.5 (see Bennett et
18 al, Nature Genet 1995, 9:284-292). A VNTR with a 40 bp
19 repeating sequence in the dopamine transporter gene,
20 which is closely related to the serotonin transporter
21 gene, has been suggested to play a role in determining
22 susceptibility to some forms of alcoholism (see Perisco
23 et al, Biol Psychiatry 1993, 34:265-267 and Goldman
24 Nature Med 1995, 1:624-625).

25
26 There are several possible mechanisms by which
27 variation in the VNTR in the serotonin transporter gene
28 might influence susceptibility to affective disorders.
29 Variations in the VNTR region may play a role in
30 regulation transcription, possibly through an adjacent
31 AP-1 motif (see Lesch et al, 1994, supra). Variations
32 in the VNTR at the *IDDM2* locus have been shown to
33 influence the expression of insulin mRNA in pancreatic
34 cell lines: gene constructs containing haplotypes of
35 the VNTR which confer susceptibility to type 1 diabetes
36 are expressed at higher levels than other haplotypes

1 (see Lucassen et al, Hum Mol Genet 1995, 4:501-506).
2 Alternatively, the polymorphism may be in linkage
3 disequilibrium with a susceptibility locus nearby, as
4 is the case for alleles of a VNTR downstream of the
5 human phenylalanine hydroxylase gene (see Goltsov et
6 al, Am J Human Genetics 1992 51: 627-636).

7

8

9 Example 2

10 The preliminary study described in Example 1 was
11 expanded.

12

13 Subjects. The design of the expanded study was
14 approved by the relevant committee for Medical Ethics.

15

16 One hundred and nineteen individuals with single or
17 recurrent major depressive episodes and 128 individuals
18 with bipolar disorder were compared with a group of 346
19 controls. These totals include 39 unipolar, 44 bipolar
20 and 193 controls from our preliminary study (described
21 in Example 1). Patients with major affective disorder
22 were recruited from the in-patient and out-patient
23 population of the Royal Edinburgh Hospital. All
24 patients met DSM III-R criteria for major depressive
25 disorder or bipolar disorder and also the probable
26 Research Diagnostic Criteria according to the Schedule
27 for Affective Disorders and Schizophrenia (Lifetime
28 version) (SADS-LA) (Endicott and Spitzer 1978, Archives
29 of General Psychiatry 35 : 837-844). Control samples
30 were obtained from two sources: 103 volunteers who were
31 screened to exclude past psychiatric illness by a brief
32 interview and 243 anonymous donors from the Scottish
33 Blood Transfusion Service who met usual criteria for
34 blood donation and were therefore not currently on any
35 psychotropic medication.

36

1 The methodology was as described above for Example 1,
2 and a minimum of 15 examples of each allele were
3 directly sequenced.

4
5 Statistical Methods. In addition to the Chi squared
6 test and Fisher exact test (two tailed), a comparison
7 of allele frequency distributions between the control
8 and patient groups was made by multiple analysis of
9 variance (MANOVA) using the Statistical Package for the
10 Social Sciences (SPSS Apple Macintosh v 4.0).
11 Bonferroni correction was applied to allow for multiple
12 comparisons when the Chi squared test was employed to
13 compare the affective sub-groups with controls. Odds
14 ratios and confidence limits were calculated by
15 standard methods.

16
17 Characterisation of the VNTR alleles supported the
18 results reported in Example 1. The 15 examples of each
19 allele sequenced proved to be identical and supported
20 the consensus sequence and sequence of repeats reported
21 in Example 1.

22
23 Association Study. Table 2 illustrates the
24 distribution of genotypes and allele frequencies for
25 the VNTR in the control and patient samples. The
26 distributions of genotype and allele frequencies were
27 similar in the total control and patient samples
28 compared to those described for the preliminary study.

29
30 There was a significant difference between patients
31 with affective disorder and controls in the proportion
32 of individuals carrying the STin2.9 allele (Table 2).
33 This was true for both unipolar and bipolar sub-groups
34 although there appeared to be a larger effect in the
35 unipolar group (Table 2). For the risk of unipolar
36 disorder given a single STin2.9 allele, the odds ratio

1 was 4.44 (95% CI, 1.65-11.95) and for bipolar disorder
2 3.22 (95% CI, 1.15-9.09).

3

4 The mean age of the volunteer controls was 45.04 (SD
5 15.21) and of the patients 41.23 (SD 15.00). There was
6 no significant sex difference in the distribution of
7 STin2.9 allele between patient and control groups
8 ($\chi^2=0.99$).

9

10 Allele frequencies were also calculated for the control
11 and patient groups (Table 2). MANOVA showed a
12 significant difference in overall allele distribution
13 between the affective disorder group v control group
14 and the unipolar v control group (Table 2). There was
15 a similar trend in the bipolar sample which did not
16 reach statistical significance ($p=0.065$, 2 d.f., two
17 tailed).

18

19 Discussion.

20 There was a significant overall difference between
21 affective disorder and control groups in the frequency
22 distribution of alleles of the human serotonin
23 transporter gene. The main finding is a significant
24 increase in the frequency of the STin2.9 allele in
25 patients with major affective disorder. This extends
26 the previous finding described in Example 1 to a larger
27 patient and control samples from the same population.

28

29 The structure of the VNTR consisted of 9, 10 or 12
30 copies of a 16-17 bp motif. The three alleles
31 contained seven variants of the repetitive element
32 (indicated as A-G in Figure 1) in a specific order. We
33 did not detect any allele containing 11 repeats, even
34 though it has been reported in another study that the
35 majority of chromosomes examined contained either 10 or
36 11 copies (Lesch et al, 1994, Journal of Neural

1 Transmission 95 : 157-162). STin2.10 is similar to the
2 10 repeat allele described by Lesch et al, 1994 supra
3 although repeats A and D show slight sequence variation
4 and the order of elements seen here is ABCDEFDEDF
5 rather than ABCDEEEDF.
6
7 Comparison of the STin2.9, 10 and 12 alleles suggests
8 that the shorter forms may have been generated by loss
9 of central repeating elements. Evidence from VNTRs
10 such as those in the collagen type II (COL2A1) and
11 Apolipoprotein B genes suggest that the secondary DNA
12 structure may be important in the generation of new
13 alleles (Berg and Olaisen, 1993, Genomics 16 : 350-354;
14 Desmarais et al, 1993, Nucleic Acids Research 21 :
15 2179-2184). The sequences of VNTRs may favour the
16 formation of hairpins and loops, which could result in
17 the formation of new alleles by replication slippage.
18
19 The particular association between the occurrence of a
20 STin2.9 allele and the risk of affective disorder
21 requires explanation. The level of serotonin
22 transporter gene transcription may be influenced by the
23 sequences of the repetitive elements. VNTRs close to
24 the insulin (IDDM2 locus) and HRAS1 genes bind
25 transcription factors and show allelic variation
26 associated with disease (Catignani Kennedy et al, 1995,
27 Nature Genetics 9 : 293-298; Green and Krontiris, 1993,
28 Genomics 17 : 429-434). These VNTRs regulate
29 transcription in a cell and promoter specific way and
30 small differences in nucleotide sequence influence the
31 level of transcriptional activity. At the IDDM2 locus,
32 the absence of a single 14 bp repeat element designated
33 "e" has been suggested to cause loss of a protective
34 effect against the development of insulin dependent
35 diabetes (Bennett et al, 1995, Nature Genetics 9 : 284-
36 292). By analogy, the absence of the 16bp element "F"

1 near the centre of the VNTR may also have functional
2 consequences. Alternatively it may simply be the
3 overall length of the VNTR which is adjacent to a
4 putative transcription factor (AP-1) binding site, that
5 is important.

6
7 These findings support that hypothesis that allelic
8 variation in the serotonin transporter gene may
9 contribute to susceptibility for both major depression
10 and bipolar disorder.

11
12 Example 3

13 This example investigates the role of allelic variation
14 in the human serotonin transporter gene (HSERT), and in
15 particular the variable number tandem repeat (VNTR)
16 polymorphism in the second intron of the gene in
17 individuals with MO, MA, MO+MA and unaffected controls.

18
19 Subjects and Methods. Subjects were obtained by
20 screening all 40 year olds drawn from the population in
21 a region outside Copenhagen using the Danish Central
22 Person Registry, in collaboration with Russell and
23 colleagues. This sample represents a unique group of
24 migrainous individuals from what is effectively an
25 epidemiological catchment area. Seventy-six
26 individuals with MA alone and 92 with MO alone were
27 included. Eighteen individuals with co-occurrence of
28 both MO and MA were also included (see Russell et al,
29 1988, supra). For later analysis, this co-occurrence
30 group was treated both independently and as part of the
31 "combined MA" and "combined MO" groups. Forty-eight
32 controls drawn from the Danish population who had been
33 screened by a neurologist to rule out any personal
34 history of migraine were included. In addition a group
35 of 103 Scottish volunteer controls who had been
36 screened by questionnaire to exclude a personal history

1 of migraine were also included.

2

3 Methods

4 DNA Extraction and Polymerase Chain Reaction Analysis

5 Venous blood samples were obtained from the study
6 sample in EDTA vials, and were frozen immediately.
7 They were stored at -80°C prior to DNA isolation.
8 Genomic DNA was isolated as described by Smith et al
9 (see The Lancet, 1992, 339 : 1357-7). Briefly the
10 procedure was as follows:

11

12 DNA Isolation: 100 μ l venous blood was placed in a
13 microcentrifuge tube and washed in 750 μ l TE buffer by
14 thorough mixing and centrifuging at 14,000g for 2
15 minutes. The supernatant was aspirated, and the pellet
16 washed a further two times with 500 μ l TE to complete
17 lysis of red blood cells. The final pellet (peripheral
18 blood leucocytes) was lysed by adding 100 μ l lysis
19 buffer containing 200 μ g/ml Proteinase K. After
20 incubation at 55°C for 20 minutes, 100 μ l of sterile
21 water was added to the crude lysate, and this was
22 heated to 98°C for 10 minutes to inactivate the
23 proteinase.

24

25 Polymerase Chain Reaction Analysis. Target DNA was
26 amplified by the polymerase chain reaction (PCR) using
27 the specific oligonucleotide primers 8224
28 (5'-GTCAGTATCACAGGCTGCCAG-3') and 8223
29 (5'-TGTTCCCTAGTCTTACGCCAGTG-3'), according to standard
30 protocols (Ogilvie et al. Lancet 1996;347:731-733).
31 Each 50 μ l PCR amplification reaction contained 3 μ l DNA
32 lysate, 1.5mM MgCl₂, 4.5 μ l 10x reaction buffer, 1% (v/v)
33 DMSO, 200 μ M each dNTP, 200ng each primer and 1.5U Taq
34 DNA polymerase. Forty-five cycles (30s of denaturation
35 at 94°C, 30s of primer annealing at 60°C, 30s of
36 polymerisation at 72°C) were performed using a Hybaid

1 Omnigene thermocycler, with initial strand separation
2 carried out at 94°C for 5 minutes. A final
3 polymerisation step of 1 minute was performed to
4 complete elongation of all amplified strands.
5
6 Amplified products were separated on 2% agarose gels,
7 excised and purified by the Wizard PCR DNA Purification
8 System. Sequencing was achieved using the ABI PRISM
9 Dye Terminator Cycle Sequencing Ready Reaction Kit with
10 AmpliTaq DNA Polymerase, FS in a Perkin Elmer Cetus
11 thermocycler (30 cycles of 30s at 96°C, 15s at 50°C, 4
12 min at 60°C) with reverse primer 8223. Extension
13 products were purified by ethanol precipitation.
14 Electrophoresis was performed on a 4.75% acrylamide and
15 urea gel run for 13 hours at a constant power of 30W,
16 using a model 373A STRETCH DNA Sequencer. Samples were
17 stored at -20°C until required.
18
19 Target DNA was amplified by the polymerase chain
20 reaction (PCR) using the specification oligonucleotide
21 primers 8224 (5'-GTCAGTATCACAGGCTGCGAG-3') and 8223
22 (5'TGTTCTAGTCTTACGCCAGTG-3'), according to standard
23 protocols (see Smith et al, 1992, supra). The primer
24 pair amplifies the region of intron 2 containing the
25 16-17 bp repetitive element (Figure 2). To distinguish
26 between alleles, fragments were separated by
27 electrophoresis through a 5% non-denaturing
28 polyacrylamide gel, and bands visualised by UV
29 transillumination of gels stained with ethidium bromide
30 (Figure 5).
31
32 Figure 5 shows PCR analysis of HSERT intron 2 in 6
33 individuals. 5% Polyacrylamide gel stained with
34 ethidium bromide is shown. Five different genotypes
35 can be identified: STin2.12/STin2.12 (300bp: lane 1);
36 STin2.10/STin2.10 (267bp: lane 2); STin2.10/STin2.12

1 (267bp+300bp: lanes 3 and 6); STin2.9/STin2.12
2 (250bp+300bp: lane 4); and STin2.9/STin2.10
3 (250bp+267bp: lane 5). M indicates the lane containing
4 DNA markers of the molecular sizes indicated.

5
6 Examples of each allele in each of the study groups
7 were directly sequenced as described above. Alleles
8 were identified and sequences constructed using
9 GeneJockey II.

10
11 Statistical analysis. Comparison of allele frequency
12 distributions between the control and patient groups
13 and analysis of genotype distribution was carried out
14 on the raw frequencies by the χ^2 test. Yate's
15 continuity correction was applied for any 2 by 2 tables
16 with cells having values less than 10. Overall allele
17 frequency distributions were compared between the
18 control and patient groups by multiple analysis of
19 variance (MANOVA). The Statistical Package for the
20 Social Sciences was used (SPSS Mac v4.0). Hardy-
21 Weinberg equilibrium of observed allele frequencies was
22 examined by χ^2 analysis.

23

24 Results

25 Characterisation of the VNTR Alleles. Three alleles of
26 the intron 2 VNTR region of human serotonin transporter
27 (HSERT) were identified in the Danish individuals
28 (Figure 5). All of the suspected STin2.9 alleles (nine
29 in total), plus six examples of each of the alleles
30 corresponding to STin2.10 and STin2.12 were sequenced
31 and proved to be identical to those described in
32 Example 1 with no differences between groups. The
33 three alleles contained respectively, 9(STin2.9),
34 10(STin2.10) and 12(STin2.12) copies of a repetitive
35 element present as seven variants (indicated as A to G
36 in Figure 1).

1 Association Study. The distribution of genotype and
2 allele frequencies for the VNTR in control and patient
3 groups is shown in Table 3a. Figures 6 and 7 show,
4 respectively, the distribution of genotype frequency
5 and allele frequency according to the group studied.
6
7 There was no significant difference in the overall
8 distribution of genotypes between the Danish and the
9 Scottish screened control groups ($\chi^2=0.56$ (3df),
10 $p=0.0906$). In view of this similarity, further
11 comparisons with the patient groups were done using
12 both the Danish controls on their own and a combined
13 group including all 151 controls.
14
15 Comparing the MO group to the combined controls, there
16 was significant increase in the frequency of
17 individuals and the STin2.12/STin2.12 genotype ($\chi^2=4.71$
18 (1df), $p<0.05$). In addition, MO patients showed a
19 significant move away from having a single copy of the
20 STin2.10 allele when compared with combined controls
21 ($\chi^2=4.07$ (1df), $p<0.05$), although clearly these findings
22 may be interdependent. This effect was also
23 significant in the "combined MO group", which showed a
24 shift in allele frequency distribution from having a
25 single copy of the STin2.10 allele ($\chi^2=6.14$ (1df),
26 $p<0.02$) to having two copies of the STin2.12 allele
27 ($\chi^2=4.80$ (1df), $p<0.05$). For the risk of MO given a
28 homozygous STin2.12 genotype, the odds ratio was 2.177
29 (95% CI 1.053-4.501) compared to the Danish control
30 group on its own. MANOVA showed a significant
31 difference in the overall allele frequency distribution
32 between the combined MO group versus combined controls
33 ($F=3.72$ (2df), $p=0.026$). This was reflected in the
34 genotype distribution of the combined MO group where
35 the frequency of STin2.10/STin2.12 individuals was
36 reduced ($\chi^2=4.75$ (1df), $p<0.05$) while the frequency of

1 STin2.12/STin.12 individuals was increased ($\chi^2=6.46$
2 (1df), $p<0.02$).

3
4 The combined MA group had a significant increase in
5 STin2.9 carriers ($\chi^2=4.69$ (1df), $p<0.05$), and for the
6 risk of MA given a single copy of STin2.9, the odds
7 ratio was 5.080 (95% CI, 1.003-25.716). If patients
8 with co-occurrence of both MO and MA were excluded,
9 there remains a non-significant trend in this
10 direction. The MA alone group showed a much lower
11 frequency of STin2.10/STin2.12 individuals than
12 combined controls ($\chi^2=6.65$ (1df), $p<0.01$). There was
13 also a significant decrease in individuals with the
14 STin2.10/STin2.12 genotype in both MA groups. However,
15 MANOVA failed to show a significant difference in
16 overall allele frequency distribution of either MA
17 group.

18
19 The group with co-occurrence of both MO and MA showed a
20 significantly different pattern of overall allele
21 frequency distribution ($F=5.34$ (2df), $p=0.006$), again
22 with a reduction in STin2.10 carriers compared to the
23 combined controls ($\chi^2=4.34$ (1df), $p<0.05$) and this
24 difference was also significant when compared to the
25 Danish controls alone (Table 3a).

26
27 Table 3b shows a parallel study with an amplified
28 population, where similar subjects were chosen from
29 Danish MO and MA sufferers. 173 individuals having MO
30 and 94 having MA were included. 18 individuals met
31 criteria for both MO and MA. The control group of 133
32 individuals comprised 85 individuals from the same
33 source as the subjects and 48 other volunteers from the
34 Copenhagen area. All participants had a clinical
35 interview and a physical and neurological examination
36 by an experienced neurological resident. The

1 operational diagnostic criteria of the International
2 Headache Society (Society HCCotIH. Cephalagia
3 1988;Supplement 17:1-96) were used. The project was
4 approved by the Danish Ethics Committee. Methods
5 previously described were employed.

6

7 Results:

8 Comparing the MO group to controls, MO patients showed
9 a significant move away from genotypes having a copy of
10 the STin2.10 allele ($\chi^2 = 5.70$, (1df), $P = 0.017$) and a
11 significant increase in the frequency of individuals
12 with genotypes having a copy of the STin2.12 allele (χ^2
13 $= 4.68$, (1df), $P = 0.031$) although the difference in the
14 overall allele frequency distribution did not reach
15 significance. In the MO group, 44.5% of individuals had
16 a homozygous STin2.12 genotype compared to 32.3% of
17 controls. For the risk of MO given a genotype
18 homozygous for the STin2.12 allele, the odds ratio was
19 1.68 (95% CI, 1.05-2.69) compared to other genotypes.

20

21 The MA group also showed a non-significant trend away
22 from carrying the STin2.10 allele ($\chi^2 = 3.29$, (1df), P
23 $= 0.07$). This was associated both with non-significant
24 increases in STin2.12 carriers ($\chi^2 = 3.01$, (1df), P
25 $= 0.083$), and in STin2.9 carriers to 6.4% compared to
26 2.3% in the controls. This latter difference, when
27 considered as the risk of MA given a single copy of
28 STin2.9, was represented by an odds ratio of 2.95 (95%
29 CI, 0.72-12.13). This increase in STin2.9 carriers in
30 the MA group was in contrast to the MO group, where
31 there was no suggestion of such a change ($\chi^2 = 0.08$,
32 (1df), $P = 0.779$).

33

34 The group with co-occurrence of both MO and MA showed a
35 significantly different pattern of overall allele
36 frequency distribution from controls ($\chi^2 = 7.39$, (2df), P

1 =0.025), reflecting a significant reduction in
2 genotypes containing the STin2.10 allele when compared
3 to controls ($\chi^2 = 3.95$, (1df), $P = 0.047$), and a
4 non-significant shift both to STin2.9 carriers (OR =
5 5.42, 95% CI, 0.84-34.90) and to STin2.12
6 homozygosity (OR = 2.62, 95% CI, 0.96-7.10) .

7 8 Discussion

9 This example confirms by sequencing the existence in a
10 non-British population of identical allelic forms of
11 the human serotonin transporter gene intron 2 VNTR to
12 those previously described. The example demonstrates a
13 difference in the allelic distribution of the VNTR
14 between individuals with co-occurrence of MO and MA, and
15 unaffected controls. In addition, an apparent
16 dissociation between individuals suffering from
17 migraine without aura and individuals suffering from
18 migraine with aura in genotype distribution at this
19 locus is demonstrated.

20
21 The data are suggestive that the STin2.10 allele may be
22 protective against the development of both types of
23 migraine. MO patients show a significant shift away
24 from carrying the ten repeat, STin2.10 allele, towards
25 having the STin2.12 allele. While the MA patients also
26 show such a trend, they exhibit both a threefold
27 increase in carriers of the rare STin2.9 allele as well
28 as a move towards STin2.12 homozygosity when compared
29 to controls. The findings regarding the group of
30 individuals with co-occurrence of both MO and MA is
31 intriguing. Such co-occurrence is rare and the group
32 is therefore small in this epidemiological sample.
33 However, the presence of a statistically significant
34 separation in overall allele distribution in this
35 group, when compared to controls, and a significant
36 reduction in genotypes with a STin2.10 allele

1 associated with both a trend to STin2.9 elevation and
2 an increase in STin2.12 homozygosity, may reflect the
3 contribution of the different alleles to each disorder
4 while also reinforcing the distinctiveness of MO and
5 MA.

6
7 MO patients show a significant shift towards the
8 STin2.12 allele, while the MA patients show a move
9 towards more STin2.9 carriers when compared to
10 controls. The HSERT VNTR polymorphism may be only one
11 of a number of genes which may mediate susceptibility
12 to migraine. It is interesting to note that the
13 segregation analysis performed by Mochi and colleagues
14 suggested the involvement of two or more genes (see
15 Mochi et al, 1993, supra), and their proposed reduced
16 penetrance model may in fact be concealing a more
17 complex pattern of inheritance. In light of the
18 proposed role of allelic variation in the serotonin
19 transporter gene as a susceptibility factor for major
20 depression, it is of particular interest that MA has
21 been shown to be the type of migraine most strongly
22 associated with depression (Breslau et al supra). It
23 is important to emphasise that patients were not
24 excluded from either control or patient groups in the
25 present study on the basis of a history of affective
26 disorder and that this could be a confounding factor.
27 Breslau and colleagues (see Breslau et al, 1991, supra)
28 have shown that the odds ratio for migraine and
29 depression co-morbidity is generally higher with MA
30 versus controls (OR=4.0; 95% CI, 2.2-7.2) than with MO
31 versus controls (OR=2.2; 95% CI, 1.2-4.0).

32
33 The findings regarding the group of individuals with
34 co-occurrence of both MO and MA is intriguing. Such
35 co-occurrence is rare and the group is therefore small
36 in this epidemiological sample. However, the finding

1 of both a trend to STin2.9 elevation and an increase in
2 STin2.12 homozygosity in the presence of a
3 statistically significant separation in overall allele
4 distribution when compared to controls ($F=5.34$ (2df),
5 $p=0.006$), may reflect the contribution of the different
6 alleles to each disorder while also reinforcing the
7 distinctiveness of MO and MA.

8
9 The differences found in the observed and expected
10 genotype distribution for the MA group may be explained
11 by Russell's observation (see Cephalalgia, 1996) of a
12 bimodal distribution in age at onset in MA patients
13 with MA, suggesting the existence of two subtypes of
14 MA. The failure of the combined patient and combined
15 control groups to meet Hardy-Weinberg equilibrium may
16 simply be due to the fact that they are an amalgamation
17 of two separate groups.

18
19 These data support the view that susceptibility to MO
20 and MA has a genetic component and that genetic
21 susceptibility may in some cases be associated with a
22 locus at or near the serotonin transporter gene. They
23 also suggest that, in particular, the group of
24 individuals with co-occurrence of MO and MA may be
25 worthy of further investigation. The apparent
26 dissociation between MO and MA with regard to patterns
27 of HSERT genotype distribution is also of interest in
28 light of the ongoing debate over whether MO and MA are
29 in fact separate disorders or merely subtypes of a
30 unitary entity. These data support the increasing
31 epidemiological evidence suggestive of a true
32 separation between the two disorders.

33

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Medical Research Council
(B) STREET: 20 Park Crescent
(C) CITY: London
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): W1N 4AL

(A) NAME: Sharon Battersby
(B) STREET: 2 Pentland Gardens
(C) CITY: Edinburgh
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EH10 6NW

(A) NAME: George Fink
(B) STREET: 23 Regent Terrace
(C) CITY: Edinburgh
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EH7 5BS

(A) NAME: Guy Manning Goodwin
(B) STREET: 74 Murrayfield Gardens
(C) CITY: Edinburgh
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EH12 6DQ

(A) NAME: Anthony John Harmar
(B) STREET: 56 Liberton Place
(C) CITY: Edinburgh
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EH16 6NA

(A) NAME: Alan David Ogilvie
(B) STREET: 6 Eden Lane
(C) CITY: Edinburgh
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EH10 4SD

(A) NAME: Christopher Albert Dale Smith
(B) STREET: 18 Gilberstoun
(C) CITY: Edinburgh
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): EH15 2QY

(ii) TITLE OF INVENTION: Screening for disorders of serotonergic
dysfunction

(iii) NUMBER OF SEQUENCES: 5

- (iv) COMPUTER READABLE FORM:
 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 150 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGCTGTGACC CAGGGTGGGC TGTGACCCGG AGTGGGCTGT GACCCGGGGT GGGCTGTGAC	60
CCGGGTGGGC TCGACCTGG GGTGGGCTGT GACCCGGGTG GGCTGTGACC TGGGGTGGGC	120
TGTGACCCGG GTGGGCTGTG ACCTGGGATG	150

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 167 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGCTGTGACC CAGGGTGGGC TGTGACCCGG AGTGGGCTGT GACCCGGGGT GGGCTGTGAC 60
CCGGGTGGGC TGCGACCTGG GGTGGGCTGT GACCTGGGAT GGGCTGTGAC CCGGGTGGGC 120
TGTGACCTGG GGTGGGCTGT GACCCGGGTG GGCTGTGACC TGGGATG 167

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 200 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGCTGTGACC CAGGGTGGGC TGTGACCCGG AGTGGGCTGT GACCCGGGGT GGGCTGTGAC 60
CCGGGTGGGC TGCGACCTGG GGTGGGCTGT GACCTGGGAT GGGCTGTGAC CCGGGTGGGC 120
TGTGACCTGG GGTGGGCTGT GACCCGGGTG GGCTGTGACC TGGGGTGGGC TGTGACCCGG 180
GTGGGCTGTG ACCTGGGATG 200

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGTTCCCTAGT CTTACGCCAG TG

22

- (2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

- (vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCAGTATCA CAGGCTGCGA G

21

Table 1. Distribution of genotypes and allele frequencies of the VNTR in control and patient groups.

Genotype Distribution (%)

	n	STin2.9/ other	STin2.10/ STin2.10	STin2.12/ STin2.12	STin2.10/ STin2.12
Combined Controls	193	2.1	14.5	33.7	49.7
-BTS	122	1.6	13.9	35.2	49.2
-Screened	71	2.8	15.5	31.0	50.7
Affective Disorder	83	8.4 ^a	21.7	31.3	38.6
-Bipolar	44	4.5	27.3	31.8	36.4
-Unipolar	39	12.8 ^b	15.4	30.8	41.0

Allele Frequency (%)

	n	STin2.9	STin2.10	STin2.12
Combined Controls	386	1.04	39.64	59.33
-BTS	244	0.82	38.93	60.25
-Screened	142	1.41	40.85	57.75
Affective Disorder	166	4.22 ^c	41.57	54.22
-Bipolar	88	2.27	45.45	52.27
-Unipolar	78	6.41 ^d	37.18	56.41

Statistically significant differences from the combined control group were as follows:

- $\chi^2 = 6.14$, $P < 0.02$. O.R. = 4.35, 95% C.I. 1.2 - 15.3. Survives Fisher exact test (two-tailed) at $P < 0.05$.
- $\chi^2 = 10.05$, $P < 0.002$. O.R. = 6.95, 95% C.I. 1.8 - 27.2. Survives Fisher exact test (two-tailed) at $P < 0.01$.
- $\chi^2 = 4.49$, $P < 0.05$. O.R. = 4.20, 95% C.I. 1.2 - 14.6.
- $\chi^2 = 6.00$, $P < 0.02$. O.R. = 6.51, 95% C.I. 1.7 - 24.9.

	N	Genotype distribution (%)						Allele Frequency (%)				F
		9+ 10 or 12	9 10	9 12	10 10	10 12	12 12	STIn2.9	STIn2.10	STIn2.12		
Controls												
All controls	346	2.02	0.29	1.73	14.74	50.87	32.37	1.01	40.32	58.67		
B.T.S.	243	2.47	0.41	2.06	15.64	49.79	32.10	1.23	40.74	58.02		
screened	103	0.97	0.00	0.97	12.62	53.40	33.01	0.49	39.32	60.19		
Patients												
All affective disorder	247	7.29 ¹	3.24	4.05	13.77	45.34	33.60	3.64	38.06	58.30	5.08 ⁴	
bipolar	128	6.25 ²	2.34	3.91	14.06	48.44	31.25	3.13	39.45	57.42	2.74	
unipolar	119	8.40 ³	4.20	4.20	13.45	42.02	36.13	4.20	36.55	59.24	5.42 ⁵	

Table 2: Distribution of genotype and allele frequencies of VNTR in control and patient groups

Significant differences from the combined control group in the total sample: ¹ $\chi^2 = 9.89$, $P = 0.0017$, 1df; ² $\chi^2 = 5.45$, $P = 0.0196$, 1df; ³ $\chi^2 = 10.23$, $P = 0.0014$, 1df; ⁴ MANOVA, 2df, $P = 0.006$; ⁵ MANOVA, 2df, $P = 0.005$.

	N	Genotype Distribution, % INI						Allele Frequency, % INI			F
		9+10	9+12	10+10	10+12	12+12		STIn2.9	STIn2.10	STIn2.12	
Controls											
Combined	151	1.32 [2]	1.32 [2]	13.25 [20]	53.64 [81]	31.79 [48]		0.66 [2]	40.07 [121]	59.27 [179]	
Scottish screened	103	0.97 [1]	0.97 [1]	12.62 [13]	53.40 [55]	33.01 [34]		0.49 [1]	39.32 [61]	60.19 [124]	
Danish screened	48	2.18 [1]	2.08 [1]	14.58 [7]	54.17 [26]	29.17 [14]		1.04 [1]	41.67 [40]	57.29 [55]	
Migraine with aura											
Combined	94	6.38 [6] ¹	2.13 [2]	15.96 [15]	34.04 [32] ^{2,4}	43.62 [41]		3.19 [6]	35.11 [66]	61.70 [116]	
MA alone	76	5.26 [4]	1.32 [1]	18.42 [14]	35.53 [27] ^{3,8}	40.79 [31]		2.63 [4]	38.16 [58]	59.21 [90]	
Migraine without aura											
Combined	110	3.64 [4]	2.73 [3]	9.09 [10]	40.00 [44] ⁴	47.27 [52] ^{5,6}		1.82 [4]	29.55 [65] ^{7,10}	68.44 [151] ¹⁰	3.72 ¹¹
MO alone	92	2.17 [2]	2.17 [2]	9.78 [9]	42.39 [39]	45.65 [42] ⁶		1.09 [2]	30.98 [57] ⁶	67.93 [125]	
Migraine with and without aura											
MO + MA	18	11.11 [2]	5.56 [1]	5.56 [1]	77.78 [5]	55.56 [10]		5.56 [2]	22.22 [8] ^{9,8}	77.22 [26]	5.34 ¹²

Table 3a. Distribution of genotype and allele frequencies of VNTR in control and patient groups

Statistically significant differences from the combined control group were as follows:

1. $\chi^2 = 4.49$, $p < 0.05$, 1df; OR = 5.000, 95% CI = 1.000-25.716
2. $\chi^2 = 8.96$, $p < 0.01$, 1df; OR = 0.446, 95% CI = 0.262-0.760
3. $\chi^2 = 6.65$, $p < 0.01$, 1df; OR = 0.476, 95% CI = 0.270-0.841
4. $\chi^2 = 4.75$, $p < 0.05$, 1df; OR = 0.574, 95% CI = 0.350-0.949
5. $\chi^2 = 6.44$, $p < 0.02$, 1df; OR = 1.974, 95% CI = 1.158-3.195
6. $\chi^2 = 4.71$, $p < 0.05$, 1df; OR = 1.800, 95% CI = 1.056-3.076
7. $\chi^2 = 6.16$, $p < 0.02$, 1df; OR = 0.627, 95% CI = 0.433-0.908
8. $\chi^2 = 4.07$, $p < 0.05$, 1df; OR = 0.671, 95% CI = 0.455-0.990
9. $\chi^2 = 4.34$, $p < 0.05$, 1df; OR = 0.627, 95% CI = 0.388-0.969
10. $\chi^2 = 4.80$, $p < 0.05$, 1df; OR = 1.504, 95% CI = 1.043-2.168
11. MANOVA, $p = 0.026$, 2df
12. MANOVA, $p = 0.004$, 2df

Statistically significant differences from the Danish screened control group were as follows:

- A. $\chi^2 = 5.33$, $p < 0.05$, 1df; OR = 0.438, 95% CI = 0.215-0.888
- B. $\chi^2 = 4.19$, $p < 0.05$, 1df; OR = 0.466, 95% CI = 0.220-0.974
- C. $\chi^2 = 4.50$, $p < 0.05$, 1df; OR = 2.177, 95% CI = 1.050-4.501
- D. $\chi^2 = 4.43$, $p < 0.05$, 1df; OR = 0.567, 95% CI = 0.357-0.967
- E. $\chi^2 = 4.28$, $p < 0.05$, 1df; OR = 0.400, 95% CI = 0.615-0.969

	N	Genotype Distribution, % [N]					Allele Frequency, % [N]		
		9+10	9+12	10+10	10+12	12+12	STn2.9	STn2.10	STn2.12
Controls	133	0.8 [1]	1.5 [2]	14.3 [19]	51.1 [68]	32.3 [43]	1.1 [3]	40.2 [107]	58.7 [156]
Migraine without aura	173	0.6 [1]	2.9 [5]	12.7 [22]	39.3 [68]	44.5 [77] ⁴	1.7 [6]	32.7 [113]	65.6 [227]
Migraine with aura	94	4.3 [4]	2.1 [2]	16.0 [15]	34.0 [32] ³	43.6 [41]	3.2 [6]	35.1 [66]	61.7 [116]
Migraine with and without aura	18	5.6 [1]	5.6 [1]	5.6 [1]	27.8 [5]	55.6 [10]	5.6 [2]	22.2 [8]	72.2 [26]

Table 3b. Distribution of genotype and allele frequencies of VNTR in control and patient groups.

Statistically significant differences from the control group were as follows:

$$1\chi^2 = 7.39, (2df), P = 0.025$$

$$2\chi^2 = 4.26, (1df), P = 0.039$$

$$3\chi^2 = 6.52, (1df), P = 0.011$$

$$4\chi^2 = 4.68, (1df), P = 0.031$$

1 Claims:

2 1 A polynucleotide having a sequence as set out in
3 any one of SEQ ID Nos:1, 2, 3, 4 and 5 or a part
4 thereof.

5

6 2 The alleles STin2.9, STin2.10 and STin2.12 as
7 described herein.

8

9 3 A vector comprising a polynucleotide as claimed in
10 claim 1 or an allele as claimed in claim 2.

11

12 4 A cell containing a polynucleotide as claimed in
13 claim 1, an allele as claimed in claim 2 or a vector as
14 claimed in claim 3.

15

16 5 A cell as claimed in claim 4, wherein at least
17 part of the polynucleotide or allele is located in
18 intron 2 of the serotonin transporter gene.

19

20 6 The use of:

21 - a polynucleotide as claimed in claim 1 or an
22 allele as claimed in claim 2 or a vector as
23 claimed in claim 3 or a derivative or a part
24 thereof; or

25 - a cell as claimed in claim 4 or claim 5;
26 in genetic engineering procedures.

27

28 7 A transgenic animal containing a polynucleotide as
29 claimed in claim 1, an allele as claimed in claim 2 or
30 a vector as claimed in claim 3.

31

32 8 A transgenic mammal containing a polynucleotide as
33 claimed in claim 1, an allele as claimed in claim 2 or
34 a vector as claimed in claim 3.

35

36 9 The use of a cell as claimed in claim 4 or claim 5

1 or a transgenic animal as claimed in claim 7 or claim 8
2 to evaluate potential agents which may be effective for
3 combatting psychiatric disorders and other disorders of
4 serotonergic function.

5
6 10 A method of evaluating the ability of an agent to
7 influence the expression of a serotonin transporter,
8 said method comprising exposing said agent to a cell as
9 claimed in claim 4 or claim 5, or to a transgenic
10 animal as claimed in claim 7 or claim 8, and
11 determining the effect of said agent on the expression
12 of the serotonin transporter gene.

13
14 11 A method of diagnosis of migraine or psychiatric
15 disorders, or of susceptibility thereto, said method
16 comprising analysing the number of VNTR repeats in the
17 second intron of the serotonin transporter gene.

18
19 12 A method as claimed in claim 11, wherein said
20 method analyses the number of copies of alleles
21 STin2.9, STin2.10 and/or STin2.12.

22
23 13 A method as claimed in claim 11 or 12, wherein the
24 disorders include aggression, dementia, alzheimer's
25 disease, mood disorders, depressive disorders, anxiety
26 disorders, personality disorders and general medical
27 disorders characterised by abnormal serotonergic
28 function.

29
30 14 A method as claimed in claim 11 or claim 12
31 wherein the number of VNTR repeats or said alleles
32 occurring in intron 2 of the serotonin transporter gene
33 is determined in vitro.

34
35 15 A method as claimed in any one of claims 11-14,
36 wherein the number of VNTR repeats or the presence of

1 said alleles is determined using polymerase chain
2 reaction, heteroduplex analysis, comparative genome
3 hybridisation, single strand conformational
4 polymorphism analysis, ligase chain reaction and/or
5 Southern blotting.

6

7 16 A method as claimed in any one of claims 11-15,
8 wherein a sample from one individual is analyzed.

9

10 17 A method as claimed in claim 16, wherein the
11 sample comprises body tissue or body fluids containing
12 DNA.

13

14

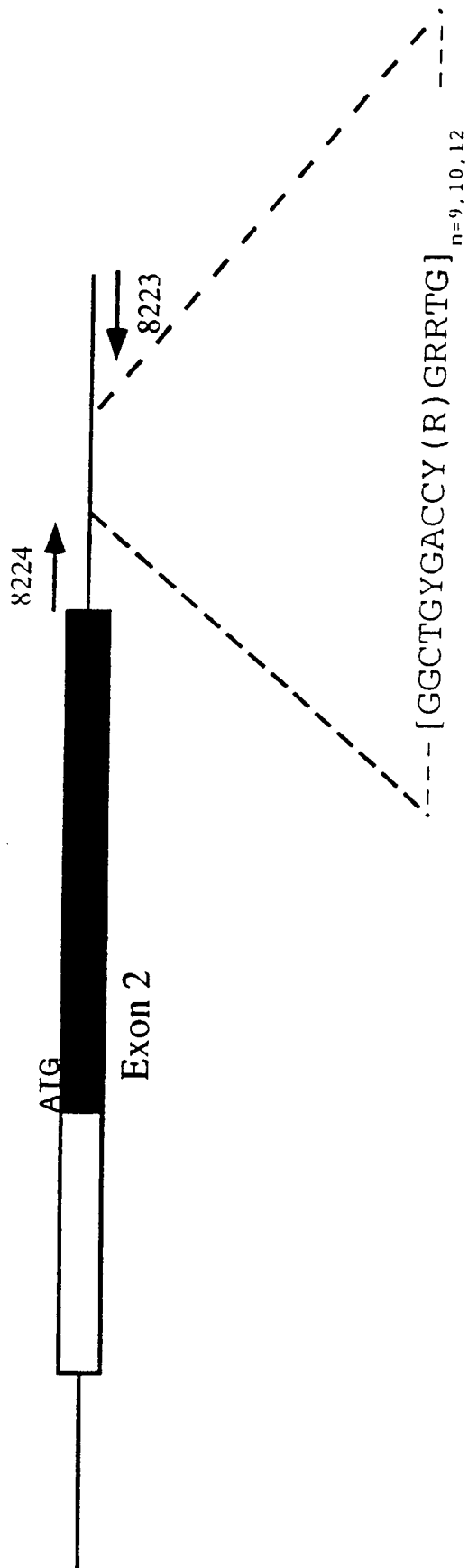
15

FIGURE 1

Lesch 10	GGCTGTGACCCGAGGTG	GGCTGTGACCCGGAGTG	GGCTGTGACCCGGGGTG	GGCTGTGACCCGGGGTG	GGCTGCGACCTGGGGTG
STin2.9	GGCTGTGACCCAGGGTG	GGCTGTGACCCGGAGTG	GGCTGTGACCCGGGGTG	GGCTGTGACCC	GGCTGCGACCTGGGGTG
STin2.10	GGCTGTGACCCAGGGTG	GGCTGTGACCCGGAGTG	GGCTGTGACCCGGGGTG	GGCTGTGACCC	GGCTGCGACCTGGGGTG
STin2.12	GGCTGTGACCCAGGGTG	GGCTGTGACCCGGAGTG	GGCTGTGACCCGGGGTG	GGCTGTGACCC	GGCTGCGACCTGGGGTG
	1(A)	2(B)	3(C)	4(D)	5(E)
Lesch 10	GGCTGTGACCTGGGGTG	GGCTGTGACCCGGGGTG	GGCTGTGACCTGGGGTG
STin2.9	GGCTGTGACCC	GGCTGTGACCTGGGGTG
STin2.10	GGCTGTGACCTGGGATG	GGCTGTGACCC	GGCTGTGACCTGGGGTG
STin2.12	GGCTGTGACCTGGGATG	GGCTGTGACCC	GGCTGTGACCTGGGGTG	GGCTGTGACCC	GGCTGTGACCTGGGGTG
	6(F)	7(D)	8(G)	9(D)	10(G)
Lesch 10	GGCTGTGACCCGGGGTG	GGCTGTGACCTGGGATG	GGCTGTGACCTGGGGTG	GGCTGTGACCTGGGGTG	GGCTGTGACCTGGGGTG
STin2.9	GGCTGTGACCC	GGGTG	GGCTGTGACCTGGGATG	GGCTGTGACCTGGGGTG	GGCTGTGACCTGGGGTG
STin2.10	GGCTGTGACCC	GGGTG	GGCTGTGACCTGGGATG	GGCTGTGACCTGGGGTG	GGCTGTGACCTGGGGTG
STin2.12	GGCTGTGACCC	GGGTG	GGCTGTGACCTGGGATG	GGCTGTGACCTGGGGTG	GGCTGTGACCTGGGGTG
	11(D)	12(G)			

Differences between STin2.10 and Lesch are : GA instead of AG in repeat 1 (underlined), our repeats 4,7,9 and 11 are 16bp long rather than 17, STin2.10 appears to be lacking repeats 7 and 8 whilst Lesch appears to lack 6 and 7.

Figure 2



SUBSTITUTE SHEET (RULE 26)

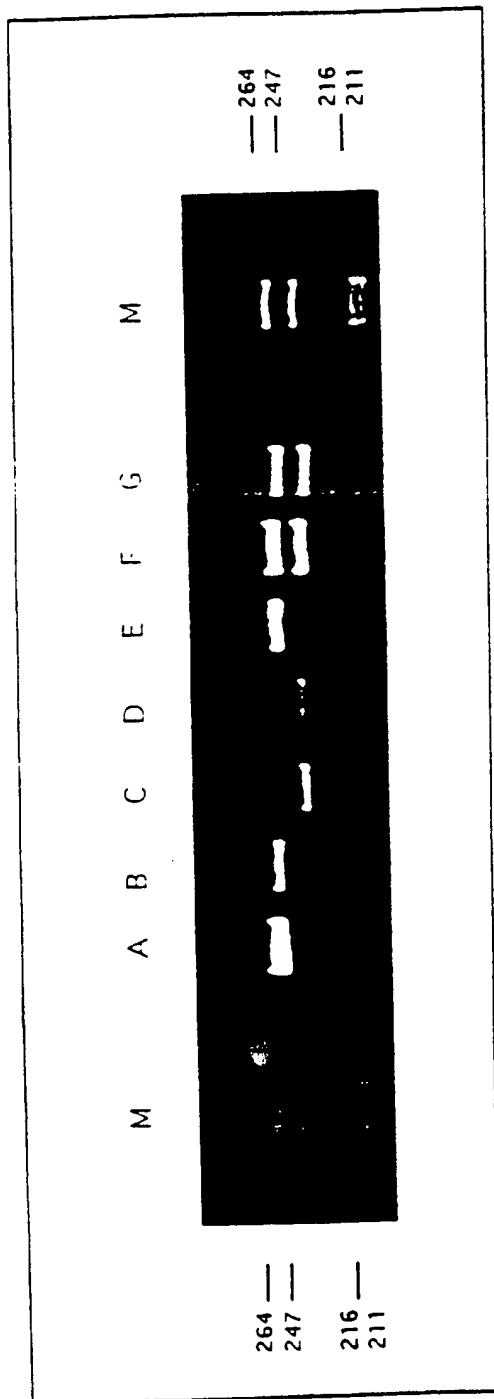
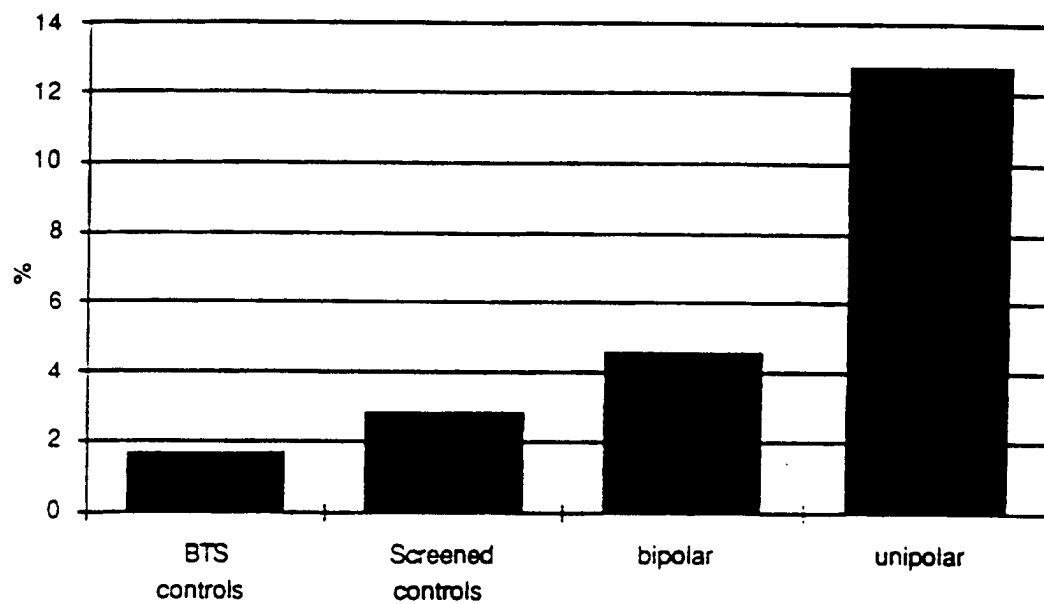


Figure 3 : Ethidium bromide stained 5% polyacrylamide gel shows PCR fragments from 7 DNA samples with 10+10 (A & E), 10+12 (B), 9+12 (C & D) and 9+10 (F & G) copies of 16 or 17 bp VNTR
M = DNA Markers

Figure 4.

SUBSTITUTE SHEET (RULE 26)

Figure 5

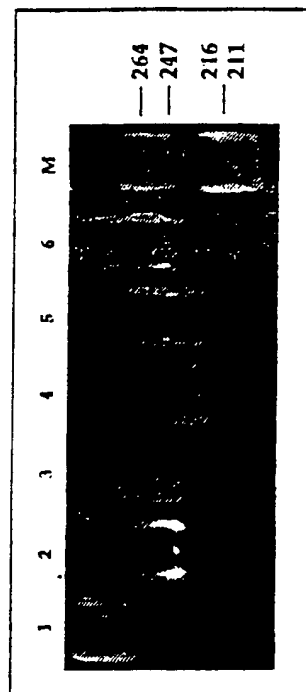


Figure 6.

6/6

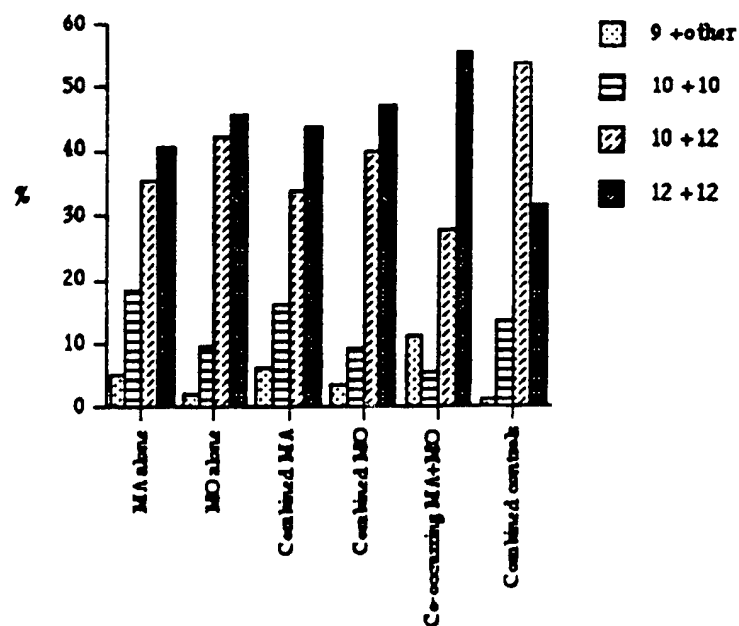
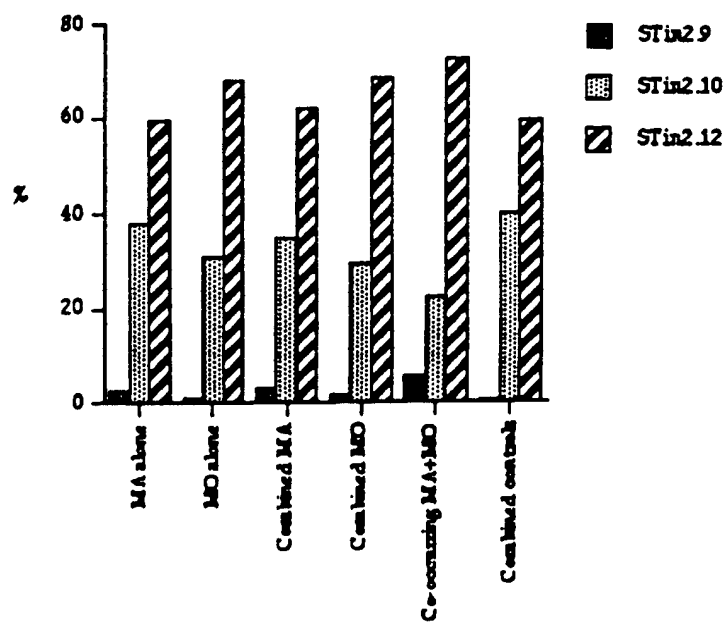


Figure 7.



INTERNATIONAL SEARCH REPORT

International Application No.
GB 96/02360

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 A01K67/00 C12Q1/02 C12Q1/68
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A01K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LANCET, MAR 16 1996, 347 (9003) P731-3, ENGLAND, XP000615491 OGILVIE AD ET AL: "Polymorphism in serotonin transporter gene associated with susceptibility to major depression." see the whole document ---	1-6
P,X	NEUROREPORT, 7 (10). 1996. 1675-1679., XP000613721 COLLIER D A ET AL: "The serotonin transporter is a potential susceptibility factor for bipolar affective disorder" see the whole document --- -/--	1-6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- * "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- * "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * "&" document member of the same patent family

Date of the actual completion of the international search

14 January 1997

Date of mailing of the international search report

29. 01. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Espen, J

INTERNATIONAL SEARCH REPORT

International Application No.
1/GB 96/02360

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J NEURAL TRANSM, vol. 95, 1994, pages 157-162, XP000613887 LESCH KP ET AL.: "Organization of the human serotonin transporter gene" see abstract; figure 1	1-6
Y	see page 158, paragraph 4 - page 159 ---	9-17
Y	WO,A,93 08261 (UNIV EMORY ;UNIV DUKE (US)) 29 April 1993 see page 13 - page 14 -----	9-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national application No.

PCT/GB 96/ 02360

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 96/ 02360

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 11-13, 15-17 (as far as an in vivo method is concerned) are directed to a method of treatment of diagnostic method practised on the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

on on patent family members

, GB 96/02360

Form PCT/ISA:210 (patent family annex) (July 1992)

